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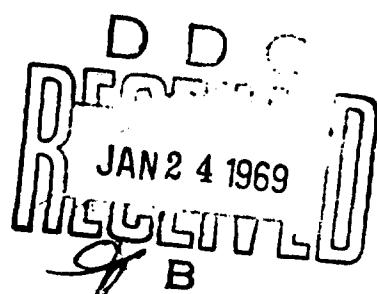
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Cultivation of yellow fever virus in human explants.

by C. Hallauer.

In 1932, Haagen and Theiler (9) were the first to grow yellow fever virus (French neurotropic strain) in cover glass and flask cultures of embryonal chicken tissue. The sustained passage of this virus strain in vitro did not lead to a significant viral transmutation (Haagen 7, Lloyd, Theiler and Ricci 14). Cultivation of the pantropic virus strain (Asibi strain) in embryonal chicken tissue failed (Haagen 8), but was later successfully carried out by a preliminary culture passage in explants of embryonal mouse tissue (Lloyd, Theiler and Ricci 14). A number of viral mutants (17 E, 17 AT, 17 D) were isolated by continuous cultivation of this virus strain in explanted mouse and chicken tissue, all of which were marked by strongly reduced viscerotropy and, partially (17 D), by decreased neurotropy (14,16).

The animal explants (mouse, chicken, monkey) used by Theiler and coworkers are not suited for primary isolation or initial cultivation of unmodified, pantropic virus. The production of culture strains is best achieved in explants of embryonal mouse brain; strains adapted to this tissue may then be maintained in explants of different types (embryonal mouse and chicken tissue) (15).

Theiler (15) grew two pantropic virus strains (French viscerotropic and J.S.S. strain) in sustained passages in vitro, utilizing the same passage and explantation techniques as those known from the transformation of the Asibi strain into 17 D. The desired goal, the production of a virus mutant optimal for immunization, was not reached, however. The immunogenic strain 17 D, marked by a high-grade mitigation of its viscerotropic and neurotropic properties, therefore represents a unique mutant which has not been reproduced to date, and for whose genesis the factors previously held to be essential (sustained culture in embryonal chicken tissue without brain and spinal cord) can hardly be held responsible (15).

It seemed desirable, in view of these circumstances, to test the behavior of yellow fever virus in human tissue explants, with emphasis on the question whether such such explants are as suitable or more suitable for the demonstration of viscerotropic and neurotropic or modified virus strains, as well as for the evaluation of immune sera, as the mouse test. The expected feasibility of growing yellow fever virus in tissue explants of its natural host also offered increased interest with respect to the question concerning the stability of strain 17 D on one hand, and the mutation of the original Asibi strain on the other. A preliminary report (10) on this investigation, in progress for over three years, has already been published.

Material and methods.

Virus strains: a) Strain 17 D of different origin (Wellcome, London; Institute Pasteur, Paris; Tropeninstitut Amsterdam).

b) French neurotropic strain (Rockefeller Institute, New York; Institute Pasteur, Paris).

c) Asibi strain (Rockefeller Institute, New York).

Explants: The tests were conducted predominantly with carcinoma strains HeLa "Minneapolis" and "Gip" and KB. To a lesser extent, the tumor strains HeLa "Geneve," "Zurich;" Detroit-6 and two strains of normal human tissue (amnion, kidney) were tested for their feasibility in virus cultivation.

Nutrient media. In the initial growth and the maintenance of HeLa strains we used media whose composition has already been described (12). All other tissue was grown on the following nutrient fluid:

Calf serum	10.0	Penicillin	500 U/cc
Lactalbumin 5%	10.0	Streptomycin	50 γ/cc
Medium 199	10.0	Nystatin (Squibb)	100 U/cc
Hanks solution	70.0	pH	7.3 to 7.5

The explanted tissue was initially grown in flasks (3.5 X 7.5 cm bottom surface), later exclusively in roll cultures (125 X 16 mm). Transplantation was carried out by the usual method of trypsinization.

Viral infection of the tissue explants. Four fully grown explants each were treated with calibrated doses of virus (diluted with nutrient) (10^{-1} to 10^{-8}) and incubated for 16 to 20 hours at 37°C. Subsequently the nutrient was changed daily and virus passage was carried out at the time of maximal cytopathogenic effect (usually on the 5th to the 7th day p.i.). For purposes of preservation, all viral samples were adjusted to pH 7.4 to 7.6 and frozen at -20°C or lyophylized.

Virus demonstration: The macroscopically and microscopically visible cytopathogenic effect permitted the determination of the 50% infection titer for the culture (KID_{50}), computed according to Reed and Muench. At periodic intervals the infectivity of the passage virus was tested simultaneously by intracerebral infection of mice (4 mice per viral dilution, 0.03 cc volume of injection).

Serological identification of the cultured virus: All cultivated virus strains were tested for specificity at intervals of 30 to 50 passages. For this purpose, 100 to 1,000 tissue culture doses of passage virus were combined at equal parts with a diluted (1:10) monkey hyper-immune serum (titer 1:100) and normal serum; the mixtures were incubated for 60 minutes at room temperature (20°C) and then transplanted to explants. The specificity of the virus-antibody reaction was established from the inhibition of cytopathogenic effect.

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Evaluation of yellow fever immune sera in explants: The virus-neutralizing titer (ND_{50}) of the available sera (1 each of a hyperimmune serum from monkeys and mice, 2 human immune sera) was again determined by inhibition on explants. Serum dilutions (1:20 to 1:1280) prepared with nutrient were mixed with a constant, positively cytopathogenic dose of virus (100 KID_{50}) and instilled in four explants each after standing at room temperature (20°C) for 60 minutes. In some cases a constant serum dilution (1:20) was tested for cytopathogenic inhibition against calibrated amounts of virus (10^{-1} to 10^{-6}).

Test results.

Table 1 gives a perspective on the types of explant and the number of passages during which the various yellow fever strains were maintained.

Tumor cell strains HeLa and KB showed optimal suitability for sustained cultivation. No difficulties were encountered in the growth of all virus strains (17 D, French neurotropic, Asibi) in these explants, and the infection titers obtained from the initial cultures corresponded to values established in mouse tests —— at least, as far as the non-viscerotropic strains are concerned. Investigations, especially the quantitative evaluation of virus, were therefore limited primarily to virus strains preserved in these cellular substrates.

The carcinoma cell strain Detroit-6 proved inferior in the cultivation of yellow fever virus, in spite of luxuriant growth in vitro; primary cultivation of the pantropic strain Asibi failed and the transplantation of viral passage strains (17 D, Asibi) from HeLa and KB explants yielded strongly fluctuating virus titers and thereby impeded sustained passage. Similar experiences were gained earlier in work with this cell strain and classic chicken cholera virus (13).

Strain 17 D was carried through 15 passages in explants of human amnion. Further utilization of this cell strain (isolated by Dr. U. Krech, Bern) was suspended, however, since the infection titers of the various culture passages revealed considerable fluctuations and the cytopathogenic effect frequently was unduly protracted and incomplete, causing the explants to retain chronic infection for weeks, without noteworthy cellular injury and only scant viral multiplication. The tendency of the utilized amniotic strain to latent, chronic infection was expressed also in the cultivation of classic and atypical fowl cholera virus (13,11). No immediate answer can be offered to the question whether this behavior is due to the mixed cellular nature of amniotic explants or an effect of the massive viral dosage to which these explants were invariably subjected.

Attempts to grow yellow fever virus (strain 17 D) in a passage cell strain (AHNB) of the human kidney were completely unsatisfactory. After this cell strain had proved to be just as unsuitable for the cultivation of classic chicken cholera virus (13), the tests were limited to 10 passages, especially since this cell strain gave signs of tissual changes indicating contamination with a "foamy agent."

First test series: Cultivation of yellow fever virus strains (17 D and French neurotropic) in HeLa cell explants.

Transfers of four lyophylized, immunogenic strains 17 D and two neurotropic virus strains (in the form of infectious mouse brains) to HeLa cell explants of the four strains "Minneapolis," "Oip," "Zurich" and "Geneve" permitted the demonstration of virus already in initial cultures. Similarly, the sustained passage of all virus strains failed to present any difficulties whatever. No dissimilarities were found between the utilized virus strains with respect to the type of cytopathogenic effect and the degree of virus propagation.

Cytopathogenic effect. The first signs of tissual damage -- in the form of foci of strongly granulated round cells -- appear in massively infected explants (ID_{50} log 5.0 to 6.0) 3 days p.i. at the earliest and on the 5th to 6th day p.i. in less heavily infected cultures (ID_{50} log 1.0 to 4.0). Subsequently, the necrotization of tissue makes rapid progress in most cases; the nutrient medium becomes turbid due to massive expulsion of dead round cells and the cellular layer still adhering to the glass takes on the appearance of "moth-eaten" tissue. Judging from the accelerated change of the phenol red indicator, the cellular metabolism apparently is intensified during the phase of high virus propagation in comparison to control cultures; this activity is frequently arrested within a few hours, however, even before tissue destruction has reached its apex. The virus-specific nature of the cytopathogenic effect was confirmed for all viral passage strains in inhibition tests with yellow fever immune sera.

Degree of virus propagation. The infectivity titers derived from HeLa cell explants lie between 10^5 and 10^6 for the explant and the white mouse, if the explants are infected with moderate or small virus dosages. Massively infected cultures frequently yield lower infectivity titers ($\leqq 10^{-4.5}$), especially when consistently large infective doses are carried over to the explants in virus passages.

Second test series: Cultivation of yellow fever strains (17 D and Asibi) in KB explants.

Three virus strains were maintained by sustained passage through explants of the human carcinoma strain KB (Eagle):

a) 17 D (London) after 99 HeLa passages;

b) 17 D (Amsterdam), starting material: lyophylized immunogenic inoculum, charge 94/4;

c) Asibi strain (New York), starting material: lyophylized monkey serum.

The exceptional suitability of the KB strain for the demonstration of yellow fever virus was expressed in the great primary sensitivity of these explants toward the starting strains, the high infectivity of the

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passage virus and the extensive agreement of titer values determined in explants and mouse brain tests.

The cytopathogenic effect is easily seen macroscopically in the turbidity of the nutrient and the ensuing tissucal defects ("plaques"), and also microscopically. In contrast to HeLa cell explants, the cellular metabolism is generally preserved up to total destruction of the explanted tissue. No important differences were found in the cyto-pathogenic effect of strains 17 D and pantropic strain Asibi.

Chronological progression and degree of virus propagation. The course of virus multiplication was evaluated during certain passages in daily withdrawn cell-free medium of the same explants as well as by daily treatment (repeated freezing and thawing) of a complete culture from a series of simultaneously and identically infected explants from the 1st to the 10th day p.i. The determination of infectivity was carried out in four explants per virus dilution. Titration results are listed in Table 2.

As is evident, demonstration of virus in the first 2 to 3 days p.i. is possible only in decomposed tissue, and here only in relatively small amounts; the non-cellular medium is still free of virus in this early phase of infection.

The culmination point of viral propagation for strain 17 D lies between the 4th and 6th day p.i. and coincides chronologically with the first demonstration of an intense cytopathogenic effect (clouding of the medium, appearance of plaques). The virus titer drops steadily with increasing tissue destruction; surviving and virus-forming cells as a rule are absent from the 8th to the 10th day p.i.

The Asibi strain (lyophylized virus after $3\frac{1}{2}$ and $5\frac{1}{2}$ years of preservation), in comparison with 17 D virus, showed a cytopathogenic effect protracted by 3 to 4 days during the first three culture passages and destroyed the explanted tissue only partially with formation of small (0.5 to 1.0 mm), round, sharply defined plaques, so that the explants frequently showed large areas of vital cells 10 to 12 days p.i. Virus propagation was also delayed chronologically —— keeping pace with slowly advancing tissue destruction —— and showed a slightly pronounced maximum on the 6th or 7th day p.i. In later culture passages the Asibi strain behaved exactly like the 17 D strains, both with respect to the rapidity and completeness of the cytopathogenic effect and the level of the infectivity titer.

The infectivity titers (KID_{50}) for each of the three virus passage strains determined in 40 culture passages, are listed in Table 3.

Comparative virus titration in the explant and the mouse test. The degree to which the infectivity titers of explants and mouse tests coincide, was determined both for the initial virus and the culture passage virus of strains 17 D and Asibi. The comparative evaluation generally was conducted simultaneously by transplanting viral dilutions

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produced in the nutrient (usually by decimal powers, occasionally by powers of four) to 4 roll cultures each (0.25 cc to 2.25 cc medium) and 4 mice each (0.03 cc intracerebrally). Observation was continued for 10 to 12 days in the case of cultures, and 20 days in the case of mice. The titration results obtained are reproduced in Table 4.

KB explants were repeatedly proved to possess the same sensitivity as the cerebral mouse test, making them suitable for the initial demonstration of 17 D strains (as well as the neurotropic Dakar strain). This parallelism apparently is not valid for the original Asibi strain (lyophylized monkey virus after $5\frac{1}{2}$ years preservation); at any rate, the mouse test yielded a higher (by 2.5 $\log_{10}$) infectivity titer than the explant. During another evaluation of freshly prepared monkey serum, the difference in titer, although still extant, was considerably lower (1.3 $\log_{10}$).

A surprisingly close agreement in titer values (explant versus mouse) was obtained from the culture passage virus of both 17 D (London) and the Asibi strain. Exceptions were found only in the case of a few virus samples of the Asibi strain (13th, 30th and 36th passage), whose infectivity titers were considerably lower in the mouse test (by 1.3 to 2.1 $\log_{10}$) than in the explant. These titer differences probably are attributable to the fact that simultaneous evaluation could not be maintained in this case, and the mouse test titration was carried out later, after conservation for 10 to 15 days at $-20^{\circ}C$.

The extreme discrepancy in titers (log 6.3/log 1.0: explant/mouse) obtained for the culture virus of strain 17 D (Amsterdam) in the second passage undoubtedly is genuine, since this virus strain, grown exclusively in KB explants, lost its pathogenicity for mice completely after a few culture passages. In spite of that, the infectivity for mice of this culture strain was maintained throughout the entire passage through KB explants, as demonstrated by intracerebral tests of immunity, usually on the 20th day following preliminary treatment. As a rule all animals infected with viral dilutions up to the titer established in the explant, survived, resulting in close agreement between titers obtained from explants and mouse tests. Such latently immunizing infections were not seen in surviving mice in connection with the remaining viral strains (17 D London, Asibi) *).

Antibody titration in the explant. As already mentioned, the cytopathogenic effect of yellow fever virus is neutralized specifically by addition of the corresponding immune serum. This offered a possibility for the evaluation of yellow fever immune sera in explants.

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*) As determined later, strain 17 D (London) also lost its pathogenicity for mice after 99 HeLa and 44 KB passages and effected a latently immunizing infection.

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Of a total of 12 tests with 4 immune sera (2 hyperimmune sera of monkeys and mice, and 2 human sera after immunization with 17 D), the majority (8 tests) were aimed at determining the serum dilution (1:20 to 1:2560) capable of neutralizing a constant amount of virus (100 to 1000 KID_{50}, strain 17 D); a smaller number (4 tests) served to establish the viral concentration whose cytopathogenic effect was inhibited by a constant serum dilution (1:10 to 1:20). The neutralizing effect of the immune serum was evaluated not only on the basis of the suppression of cytopathogenicity, but also by negative demonstration of virus in the explant and in the cerebral mouse test.

Both methods of evaluation permitted the estimation of an immune serum's efficacy and yielded reproducible neutralization titers and indices, as proved in a number of parallel observations. The standardization of antibody titration in explants as well as comparative testing with the customary methods of neutralization in the mouse test, must await further investigation.

Mutation of the culture virus. The cultural behavior of all viruses remained unchanged by sustained passage through HeLa and KB explants. Similarly, the pathogenicity of strain 17 D (London) for mice remained constant during 141 HeLa-KB passages and that of strain Asibi for 40 KB passages, both with respect to the titer (LD_{50}) and the average lethal time (*). On the other hand, strain 17 D (Amsterdam) became apathogenic for mice after a few passages through KB explants and retained this characteristic during the entire culture passage. The infectivity or the immunizing capacity for mice, on the other hand, was almost completely retained by this culture virus strain, as already noted.

Passage virus strains 17 D London (99/40 HeLa-KB passages), 17 D Amsterdam (39 KB passages) and Asibi (36 KB passages) were used in the testing of pathogenicity for monkeys (M. rhesus). Without anticipating the results of tests now in progress, it seems certain that sustained passage through human tissue explants did not lead to reactivation of viscerotropic properties in the 17 D strains, and that the Asibi strain was deprived of its original viscerotropy.

Discussion of test results.

In 1956, Eagle, Habel, Rowe and Huebner (3) attempted to grow yellow fever virus (**) in explants of the KB cell strain. Viral propagation in continuous culture passages could not be demonstrated,

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(*) However, after the 44th KB passage, strain 17 D (London) behaved like strain 17 D (Amsterdam) in the mouse test; cf. preceding foot note.

(**) The publication does not list the virus strain.

however. Still, a partial cytopathogenic effect was observed in the primary cultures between the 5th and 7th day p.i., and virus was demonstrated in the second culture passage (although in the relatively small amount of log 2.5 ID_{50}), a result that, according to our own experience, would correspond to the behavior of a pantropic virus.

Doherty (2) recently cultivated strain 17 D successfully in explants of stabilized cell strains of human conjunctival and appendicular tissue. The findings that the cytopathogenic effect in these normal tissues has a protracted or incomplete course (appendix) or fails to appear altogether (conjunctiva), and that infected explants regenerate and expulse virus for some time, agrees extensively with personal observations of the behavior of yellow fever virus in human amniotic explants. Explanted tumor tissue (HeLa, KB) did not reveal such chronic infections, and viral propagation consistently leads to complete destruction of the explant. The assumption that neoplastic tissue is more susceptible to virus is suggested thereby, confirming the special affinity of yellow fever virus for a transplantable mouse sarcoma, as noted by Findlay and MacCallum (5), now applicable also to explanted tissue of human origin.

The degree to which the demonstration of virus and antibodies in explants is capable of replacing the mouse test, has not been clarified to date. The present studies and those of Doherty (2) already justify the assumption that tissue explants will find beneficial application in yellow fever diagnosis and research, similarly to other types of virus.

The changes in pathogenicity suffered by various strains of virus during sustained passage are interesting from several points of view. First of all it is noteworthy that one 17 D strain (Amsterdam) completely lost its pathogenicity after a few culture passages through KB explants (*), while the other strain (London) preserved its original degree of pathogenicity for mice after 141 HeLa-KB passages or a duration of over $2\frac{1}{2}$ years (see the supplemental foot note on p. 6). The assumption that the variable behavior of these passage strains is based on the divergent quality of the parental virus, cannot be excluded as long as control tests with additional 17 D strains are unavailable. On the other hand, the conception of viral mutation due to the type of explanted tissue in which virus growth is initiated, is well founded. Apparently the selection of virus particles deciding the ultimate quality of the passage virus takes place already in the initial cultures (cf. Hallauer and Kronauer, 13).

(*) Inapparent and immunizing infections of mice cerebrally inoculated with 17 D virus have been demonstrated by Fox (6) and, recently, by Collier et al. (1). Such behavior was independent of the infective dosage, however, and especially of the quality of certain immunogenic strains (17 DD low, 17 D_3). 17 D strains completely apathogenic for mice (in the sense of the viral variant described) apparently have not been discovered to date.

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It is further deduced from tentative results of pathogenicity tests with monkeys, that strain 17 D had not lost its original quality after more than 145 passages through human explants. A reversion to the viscerotropic form, such as the one apparently achieved by Findlay and Clarke (4) b monkey liver passage of the French neurotropic strain, could not be demonstrated and was not be expected a priori. The loss of viscerotropy shown by the pantropic Asibi strain is noteworthy because it proved the independence of this mutation from the species-specific origin of the explanted tissue, i.e. it takes place also in tissue explants of the natural host, (*)— experiments now in progress are aimed at establishing the degree of attenuation of the Asibi strain induced by sustained passage through KB explants.

Tables.

Table 1. Cultivation of yellow fever virus in human explants. Anzahl Passagen – number of passages. Stamm – strain. Mensch – human. Niere – kidney. Abzweigung – branch.

Table 2. Virus propagation ($\log KID_{50}$) in KB explants in relation to time (days p.i.) Tag – day. Zellfreies Medium ein und derselben Kultur – cell-free medium of the same culture. Total Kultur (Medium & Gewebe – total culture (medium & tissue). Virus im zellfreien Medium nicht nachweisbar – no virus in cell-free medium.

Table 3. Infectivity titer ($\log_{10} KID_{50}$) of yellow fever strains passed through KB explants. Impfstamm – immunogenic strain. Nicht geprueft – not tested. Nach $3\frac{1}{2}$ jaehriger Konservierung – after preservation for $3\frac{1}{2}$ years.

Table 4. Comparative titration of yellow fever strains 17 D and Asibi in KB explants and in cerebral mouse tests. Ausgangsvirus – parental virus. Zahl in Klammern – figure in brackets. Recentes Affenserum nach 5 Tagen – recent monkey serum after 5 days. Lagerung bei –80°C – storage at –80°C. Virusproben nach 10- bis 15taegiger Konservierung bei –20°C – virus samples after preservation at –20°C for 10-15 days.

(*) This conclusion applies also to the production of apathogenic mutants of classic and atypical fowl cholera virus (11,12,13).